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Host DNA Damage Response Factors Localize to Merkel Cell Polyomavirus DNA Replication Sites To Support Efficient Viral DNA Replication

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ABSTRACT

Accumulating evidence indicates a role for Merkel cell polyomavirus (MCPyV) in the development of Merkel cell carcinoma (MCC), making MCPyV the first polyomavirus to be clearly associated with human cancer. With the high prevalence of MCPyV infection and the increasing amount of MCC diagnosis, there is a need to better understand the virus and its oncogenic potential. In this study, we examined the relationship between the host DNA damage response (DDR) and MCPyV replication. We found that components of the ATM- and ATR-mediated DDR pathways accumulate in MCPyV large T antigen (LT)-positive nuclear foci in cells infected with native MCPyV virions. To further study MCPyV replication, we employed our previously established system, in which recombinant MCPyV episomal DNA is autonomously replicated in cultured cells. Similar to native MCPyV infection, where both MCPyV origin and LT are present, the host DDR machinery colocalized with LT in distinct nuclear foci. Immunofluorescence *in situ* hybridization and bromodeoxyuridine (BrdU) incorporation analysis showed that these DDR proteins and MCPyV LT in fact colocalized at the actively replicating MCPyV replication complexes, which were absent when a replication-defective LT mutant or an MCPyV-origin mutant was introduced in place of wild-type LT or wild-type viral origin. Inhibition of DDR kinases using chemical inhibitors and ATR/ATM small interfering RNA (siRNA) knockdown reduced MCPyV DNA replication without significantly affecting LT expression or the host cell cycle. This study demonstrates that these host DDR factors are important for MCPyV DNA replication, providing new insight into the host machinery involved in the MCPyV life cycle.

IMPORTANCE

MCPyV is the first polyomavirus to be clearly associated with human cancer. However, the MCPyV life cycle and its oncogenic mechanism remain poorly understood. In this report, we show that, in cells infected with native MCPyV virions, components of the ATM- and ATR-mediated DDR pathways accumulate in MCPyV LT-positive nuclear foci. Such a phenotype was recapitulated using our previously established system for visualizing MCPyV replication complexes in cells. By combining immunofluorescent staining, fluorescence *in situ* hybridization, and BrdU incorporation analysis, we demonstrate that DDR proteins are important for maintaining robust MCPyV DNA replication. This study not only provides the first look into the microscopic details of DDR factor/LT replication complexes at the MCPyV origin but also provides a platform for further studying the mechanistic role of host DDR factors in the MCPyV life cycle and virus-associated oncogenesis.

Merkel cell polyomavirus (MCPyV) was discovered in 2008 in Merkel cell carcinoma (MCC), a highly aggressive form of skin cancer with neuroendocrine characteristics (1). Independent studies have subsequently found MCPyV to be clonally integrated in more than 80% of all MCC cases (1). Epidemiological surveys for MCPyV seropositivity (2) and sequencing analyses of healthy human skin (3) have shown that MCPyV is an abundant virus frequently shed from healthy human skin surfaces, suggesting that MCPyV may represent a common component of the human skin microbial flora. Immunosuppression, advanced age, and excessive exposure to UV radiation have been identified as the principle risk factors for MCC (4). Although MCC is uncommon, its incidence has tripled over the past 20 years, and the concern for MCC grows as the size of the aging population with prolonged sun exposure increases (5). To date, much of our knowledge of polyomaviruses is inferred from decades of research on simian virus 40 (SV40), which is phylogenetically distant from MCPyV and is not known to cause cancer in humans (1, 6). It is likely that much remains to be learned about the applicability of well-understood aspects of SV40 biology to the MCPyV life cycle and the oncogenic potential of MCPyV in humans.

Like other polyomaviruses, MCPyV is a small, nonenveloped virus with a circular, double-stranded DNA (dsDNA) genome of ~5 kb (7). A noncoding regulatory region (NCRR) divides the genome into early and late coding regions. The NCRR contains the viral origin of replication (Ori) and regulatory elements/promoters for viral gene transcription (8, 9). The early region encodes three proteins, namely, large T antigen (LT), small T antigen (sT),

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and the 57kT antigen (7). The late region encodes a major capsid protein, VP1, and a minor capsid protein, VP2 (10, 11).

Similar to SV40 LT, MCPyV LT is a multifunctional protein that plays an important role in viral replication and host cell cycle manipulation (12–14). It contains a number of domains that are conserved among polyomaviruses, including a retinoblastoma (Rb)-binding domain, DnaJ domain, and CR1 domain (15). LT also has an origin-binding domain (OBD) and a C-terminal helicase domain, both of which are required for initiating viral replication (8, 9, 16).

With little being known about the MCPyV life cycle, we are interested in studying how the interactions between viral proteins and the host machinery contribute to viral replication and/or MCC oncogenesis. Emerging evidence has suggested that the host DNA damage response (DDR) is targeted by a wide variety of DNA and RNA viruses. The host DDR is composed of a network of proteins that recognize and repair various types of DNA damage. The major players in this signaling cascade are two phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs), namely, the ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) kinases. While ATM is primarily activated upon double-stranded DNA breaks (DSBs), ATR is responsive to single-stranded breaks (SSBs) (17). Normally, DSBs are recognized by the heterotrimeric Mre11-Rad50-Nbs1 (MRN) complex (17), which in turn induces autophosphorylation and activation of ATM (18). Activated ATM phosphorylates the histone variant H2AX, as well as the downstream kinase Chk2, which signals through a number of effectors, leading to cell cycle arrest (17). Depending on the severity of the damage, this pathway can also promote senescence or apoptosis. In parallel, SSBs and single-stranded DNA accumulation at stalled replication forks are recognized and coated by RPA (19). After ATR is recruited and activated by RPA, it phosphorylates H2AX and the downstream kinase Chk1, which shares a number of downstream targets with Chk2. Previous research has shown that there is a significant amount of cross talk between the ATR and ATM pathways (17, 20–23).

Multiple groups have shown that polyomavirus infection or ectopic expression of polyomavirus LT proteins is capable of inducing a DDR in the host cell (14, 24–30). The mechanism by which these LT proteins activate the host DDR is less well understood, but it has been suggested that polyomaviruses utilize the DDR machinery for viral replication. For example, both mouse polyomavirus (MPyV) infection and SV40 infection have been shown to induce a DDR that is crucial for viral replication (24, 27). More specifically, ATM-mediated phosphorylation of SV40 LT is important for its ability to drive replication of the viral origin (27, 31). Recent reports of human polyomaviruses JC polyomavirus (JCPyV) (25) and BK polyomavirus (BKPyV) (26) have also shown that ATR and ATM are both activated upon infection and that they play an important role in viral genome amplification and virion production.

In a recent study, we have provided a mechanistic analysis of MCPyV DNA replication in cultured cells (12). Additional study from our lab (University of Pennsylvania) has shown that ectopic expression of MCPyV LT activates host DDR, leading to the inhibition of cellular proliferation (14). It remains unknown whether MCPyV also utilizes the host DDR machinery for optimal viral replication and/or virion production. In this study, we investigated the role of host DDR in MCPyV replication. Using U2OS cells infected with MCPyV as well as an MCPyV DNA replication system that we previously established (12), we show that MCPyV LT and components of the ATR- and ATM-mediated DDR pathways colocalize at actively replicating MCPyV foci in the nucleus. By combining immunofluorescent staining, immunofluorescence *in situ* hybridization (immuno-FISH), bromodeoxyuridine (BrdU) incorporation, and Southern blotting analyses, we demonstrate that DDR proteins are important for maintaining robust MCPyV DNA replication.

MATERIALS AND METHODS

Cell culture, cell lines, and DNA/siRNA transfection. U2OS cells were maintained in McCoy's 5A medium (Invitrogen) containing 10% fetal bovine serum (HyClone). C33A cells were maintained in Dulbecco modified Eagle medium (Invitrogen) containing 10% fetal bovine serum. For immunofluorescent staining, C33A cells were transfected at 40 to 50% confluence using the FuGENE6 transfection reagent (Promega), according to the manufacturer's instructions, and fixed at 48 to 60 h posttransfection (hpt). For Southern blotting and flow cytometry analysis, C33A cells were transfected using the calcium phosphate method (32). Small interfering RNA (siRNA) transfection was performed using calcium phosphate, as previously described (33). To detect viral DNA replication, C33A cells were pulsed with 10 μM BrdU at 44 hpt for 2 h and cultured with normal growth medium for another 2 h before acetone fixation.

Recombinant plasmid construction. The plasmids used in this study included religated MCPyV genome, pcDNA4C-MCPyV LT, pcDNA4C-MCPyV Ori, pADL*, and pT+Ori. They have been previously described (12). The origin mutant Ori350 was constructed from pcDNA4C-MCPyV Ori by site-directed mutagenesis.

Antibodies, chemicals, and siRNAs. The following antibodies were used for immunofluorescent staining: mouse anti-MCPyV LT (CM2B4; Santa Cruz), goat anti-ATR (N-19; Santa Cruz), rabbit anti-pChk1^{S317} (Cell Signaling), rabbit anti-pATM^{S1981} (Cell Signaling), rabbit anti-pChk2^{T68} (Cell Signaling), rabbit anti-Nbs1 (Novus Biologicals), rabbit anti-pRPA32^{S33} (Bethyl Laboratories), rabbit anti-RPA70 (Cell Signaling), mouse anti-Ki-67 (Dako), Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen), Alexa Fluor 594 donkey anti-goat IgG (Invitrogen), and Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen). The following antibodies were used for Western blotting: mouse anti-MCPyV LT (CM2B4; Santa Cruz), rabbit anti-ATM (Cell Signaling), rabbit anti-ATR (Abcam), mouse antiactin (Chemicon), horseradish peroxidase (HRP)-conjugated donkey antimouse (GE Healthcare), and HRP-conjugated donkey anti-rabbit (GE Healthcare). Western Lightning Plus ECL solution was purchased from PerkinElmer. Wortmannin and NU6027 were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). Control siRNA and siRNA pools targeting human ATR and ATM were purchased from Dharmacon and used as previously described (26).

MCPyV virion preparation and infection. Native MCPyV and MCPyV pseudoviruses were prepared as previously described (10), with minor modifications. Briefly, an initial seed stock of native virions was produced by transfecting 293-4T cells (which stably express the MCPyV LT and sT proteins) with the religated recombinant genome of MCPyV isolate R17b (34–36). Five days later, native MCPyV virions were harvested and purified over an OptiPrep gradient. This initial seed stock of native MCPyV virions was used to infect fresh 293-4T cells. The MCPyV-infected 293-4T cells were harvested and lysed after 5 days of infection, and the amplified native MCPyV virions were purified over an OptiPrep gradient. For experimental infection, U2OS cells were seeded in 24-well plates and incubated with native MCPyV virions at a dose of 5×10^4 MCPyV genomes per cell for 5 days.

Immunofluorescent staining. Immunofluorescent staining was performed as previously described (12). C33A cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Cells were incubated in blocking/permeabilization buffer (0.5% Triton X-100 and 3% bovine serum albumin in PBS) for 10 min at room temperature and

stained with primary antibodies (as indicated in the appropriate figure legends) at room temperature for 1 h. Cells were washed three times with blocking/permeabilization buffer and incubated with secondary antibodies for an additional hour. Cells were then counterstained with DAPI (4′,6′-diamidino-2-phenylindole) and examined with an Olympus IX81 inverted fluorescence microscope.

Immuno-FISH. Immuno-FISH was performed as previously described (12), with slight modifications. Briefly, C33A cells were fixed and stained using antibodies as indicated in the appropriate figure legends. A specific probe recognizing pcDNA4C-MCPyV Ori and a negative-control probe recognizing the human papillomavirus type 16 (HPV16) genome were labeled with biotin-dUTP (AppliChem) using nick translation. Hybridized probes were detected with a TSA biotin system (PerkinElmer) following the manufacturer's instruction.

Microscopy and image analysis. All immunofluorescent images were collected using an inverted fluorescence microscope (IX81; Olympus) connected to a high-resolution charge-coupled-device camera (FAST1394; QImaging). Images were analyzed and presented using SlideBook (version 5.0) software (Intelligent Imaging Innovations, Inc.). The scale bars were added using ImageJ software.

Southern blotting. Southern blotting was performed as previously described (12), with slight modification. For DDR inhibitor treatment, at 20 hpt, C33A cells were incubated in medium with 20 μM wortmannin or 20 μM NU6027. Drugs were refreshed every 16 h, and cells were harvested at 52 hpt. For ATR/ATM knockdown, pT+Ori and siRNA were transfected at the same time using calcium phosphate. About 30 μg total DNA was digested with DpnI and XhoI, before being subjected to Southern blotting, while 2 μg total DNA was digested with XhoI and used as a loading control.

Western blotting. Cells were lysed in lysis buffer (10 mM HEPES, pH 7.9, 500 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) by passing through a 22-gauge needle 10 times. After a 30-min incubation on ice, the soluble and insoluble fractions were separated by centrifugation at 15,000 rpm for 5 min at 4°C. The supernatants (20 μg) were resolved on an SDS-polyacrylamide gel. Membranes were blotted according to the antibody manufacturers' instructions. Western blots were developed using enhanced chemiluminescence (ECL) solution, and images were captured using a Fuji imaging system.

Flow cytometry. C33A cells were pulse labeled with 10 μM BrdU for 2 h before trypsinization. They were then fixed, permeabilized, and stained by use of an allophycocyanin BrdU flow kit (BD Pharmingen) according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (Becton, Dickinson). Data were analyzed using FlowJo software.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance with GraphPad Prism software (version 5.0). A P value of <0.05 was considered statistically significant.

RESULTS

Colocalization of host DDR proteins and MCPyV LT in nuclear foci in cells infected with native MCPyV virions. We first investigated the relationship between the host DDR and MCPyV replication in cells infected with native MCPyV virions. We began our infection studies using cells of the U2OS cell line, which, in contrast to many other cell lines, have intact ATR and ATM pathways. We have also observed an activated DDR in U2OS cells upon ectopic MCPyV LT expression (14). Native MCPyV infection efficiency was about 10 to 15% in U2OS cells, as indicated by positive immunostaining of MCPyV LT at day 5 postinfection (dpi). It has been shown that MCPyV transcription and replication are highly restricted in all cell lines so far tested (11, 35, 36). Consistent with these observations, we observed a very low level of MCPyV LT expression in U2OS cells infected with native MCPyV virions. As we published previously (14), LT typically showed a diffuse staining pattern in the nucleus, but in ~1% of MCPyV-infected cells, we were able to observe punctate LT foci in the nucleus (Fig. 1A). Interestingly, these foci showed clear colocalization with the phosphorylated H2AX (referred to as γH2AX), a classic marker of DNA damage (Fig. 1A). We also observed colocalization of LT with ATR and $pChk2^{T68}$, with the latter being an indicator for the activation of an ATM-mediated DDR (Fig. 1A). This result suggests that the components of the ATR and ATM pathways are recruited to the MCPyV LT nuclear foci. In contrast, cells infected with MCPyV pseudovirus carrying a green fluorescent protein (GFP) reporter construct instead of the MCPyV genome did not show such DDR foci (data not shown).

To rule out the possibility that the MCPyV capsid proteins are causing this phenomenon, we pseudotyped MCPyV genomic DNA into the L1-L2 capsid of HPV16, which is capable of transducing U2OS cells. Although the LT-positive cells were equally rare, we were again able to find cells that displayed LT/$pChk2^{T68}$ colocalization in the nuclear foci (Fig. 1B). In contrast, MCPyV LT and $pChk2^{T68}$ signals were not detected in the untransduced neighboring cells (Fig. 1B). These data suggest that the MCPyV genome, and not the incoming MCPyV virion proteins, is responsible for the DDR protein/LT colocalization phenotype that we observed in MCPyV-infected U2OS cells.

The MCPyV genome alone is able to induce DDR factor/LT colocalization in nuclear foci. Based on what we saw in the HPV/MCPyV pseudovirus-transduced cells, we believed that the MCPyV genome alone could also lead to DDR factor/LT colocalization in nuclear foci. We next transfected the religated MCPyV genome into U2OS cells. The transfected cells could be identified by positive LT staining (Fig. 2). In ~5% of transfected cells, we were able to see LT colocalizing with γH2AX, ATR, and $pChk2^{T68}$ in nuclear foci at day 4 posttransfection (Fig. 2). These data confirm the colocalization of host DDR components with LT expressed from the native MCPyV genome. It is important to note that, although we have previously shown that LT expression can activate the overall activity of host DDR (14), the data presented in Fig. 1 and 2 reveal the specific accumulation of the host DDR components at the LT foci in cells treated with MCPyV virions/pseudovirions/religated genomes.

Host DDR machinery colocalizes with MCPyV LT at nuclear foci in the presence of the viral origin. Although we were able to detect DDR factor/MCPyV LT colocalization using native virion infection and religated genome transfection, the rarity of cells expressing detectable amounts of LT in these systems makes them relatively intractable. In addition, with native MCPyV systems, we were not able to prove whether the DDR factors had a specific effect on MCPyV DNA replication. In our previous studies, we established a system to detect MCPyV replication complexes in C33A cells (12). Notably, the LT foci found in MCPyV-infected and MCPyV genome-transfected cells described above greatly resembled the MCPyV LT replication complexes observed in cells cotransfected with MCPyV LT and the viral origin of replication (Ori) described in our previous study (12). We therefore adopted this LT/Ori cotransfection system in C33A cells to see if the host DDR machinery is involved in MCPyV DNA replication.

We cotransfected C33A cells with a plasmid carrying MCPyV LT and a plasmid carrying the MCPyV Ori and then detected the signals of various DDR proteins. As a negative control, we examined the behavior of a plasmid carrying a mutant MCPyV Ori (Ori350) that has previously been shown to bind MCPyV LT but is

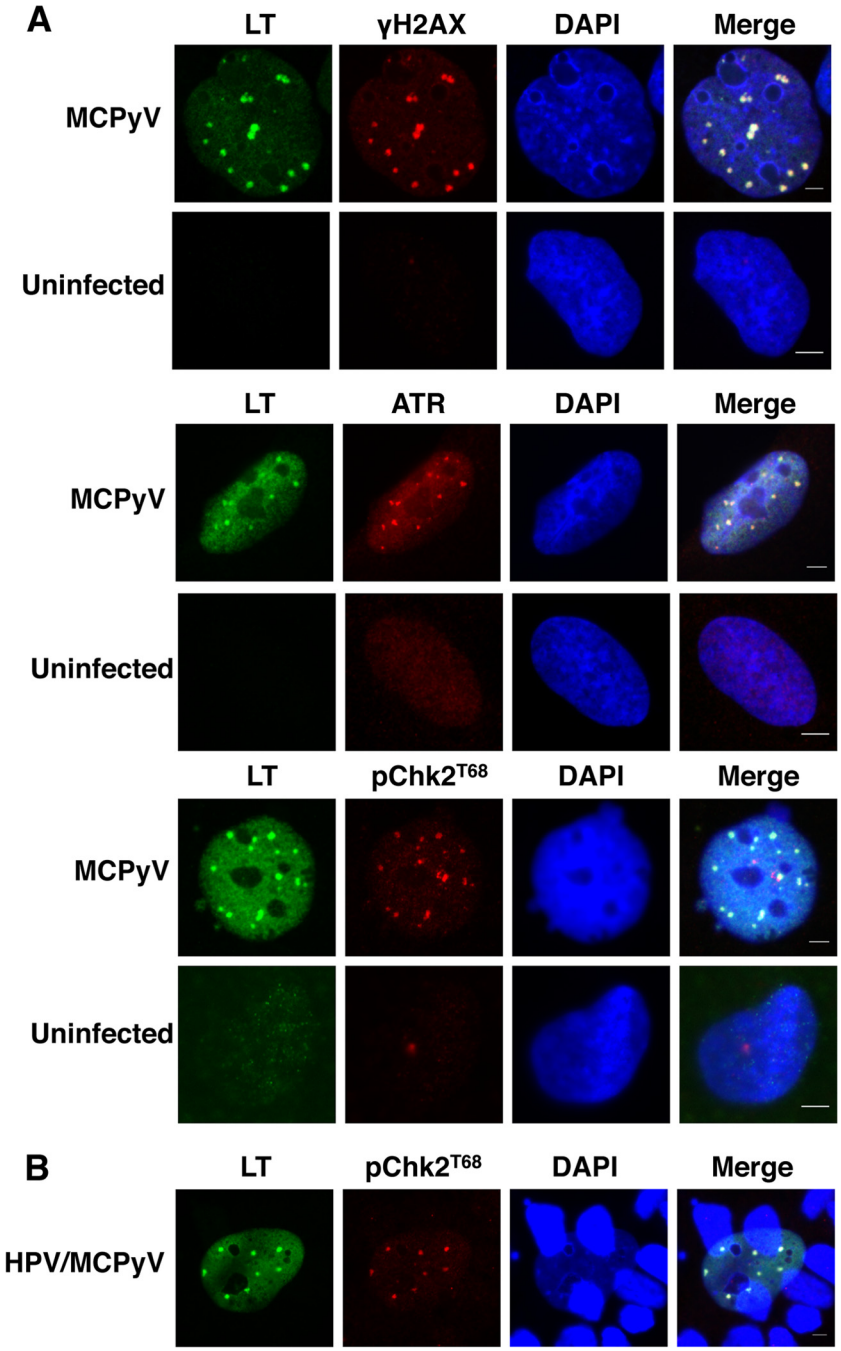

FIG 1 MCPyV LT colocalizes with γH2AX, ATR, and pChk2^{T68} in MCPyV-infected cells. U2OS cells were infected with native MCPyV virions (A) or MCPyV pseudovirus with HPV capsid carrying the MCPyV genome (B). Infected and uninfected control cells were fixed at 5 days postinfection and stained for MCPyV LT (green) and DDR proteins (red), as indicated. The cells were counterstained with DAPI. Representative pictures from at least three experiments are shown. Bars, 5 μm.

incompetent for LT-mediated replication (9). A construct with the Ori of SV40, which cannot be replicated by MCPyV LT, served as an additional negative control. MCPyV LT and most of the host DDR proteins had a diffuse nuclear pattern in the cells cotransfected with the plasmid carrying the replication-defective Ori350 or the SV40 Ori (Fig. 3 and 4 and data not shown). However, when the wild-type MCPyV Ori was present, LT formed distinct foci in the nucleus in ~20% of LT-positive cells (Fig. 3 and 4; also see Fig. 7C). This is consistent with our previous data showing that MCPyV LT formed replication foci in the nucleus in the presence of the viral Ori (12). Interestingly, multiple components of the ATM-mediated DDR were also localized at these LT foci (Fig. 3). Approximately 50 cells displaying LT foci were quantified from each of the three independent experiments. γH2AX, Nbs1, pATM^{S1981}, and pChk2^{T68} were localized at LT foci in 85.5% ± 4.0%, 87.7% ± 3.3%, 54.2% ± 13.6%, and 91.5% ± 4.4% of cells displaying LT foci, respectively.

We next investigated the other arm of the host DDR, the ATR-

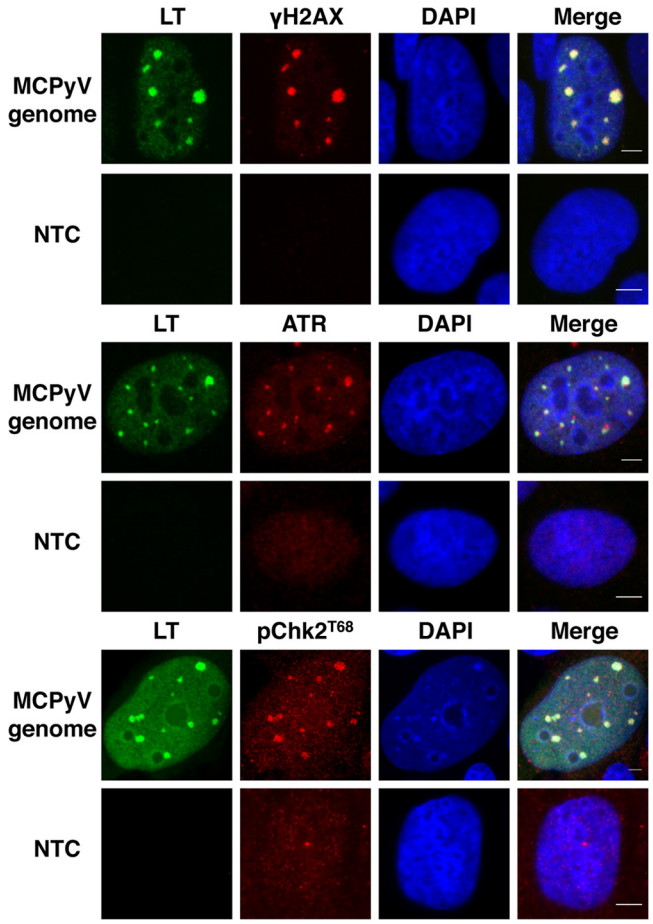

FIG 2 The MCPyV genome alone is sufficient to reproduce the LT/DDR colocalization phenotype. U2OS cells were transfected with religated MCPyV genome. Nontransfected control (NTC) cells and transfected cells were harvested at 4 days posttransfection and stained for MCPyV LT (green) and DDR proteins (red), as indicated. The cells were counterstained with DAPI. Representative pictures from at least three experiments are shown. Bars, 5 μm.

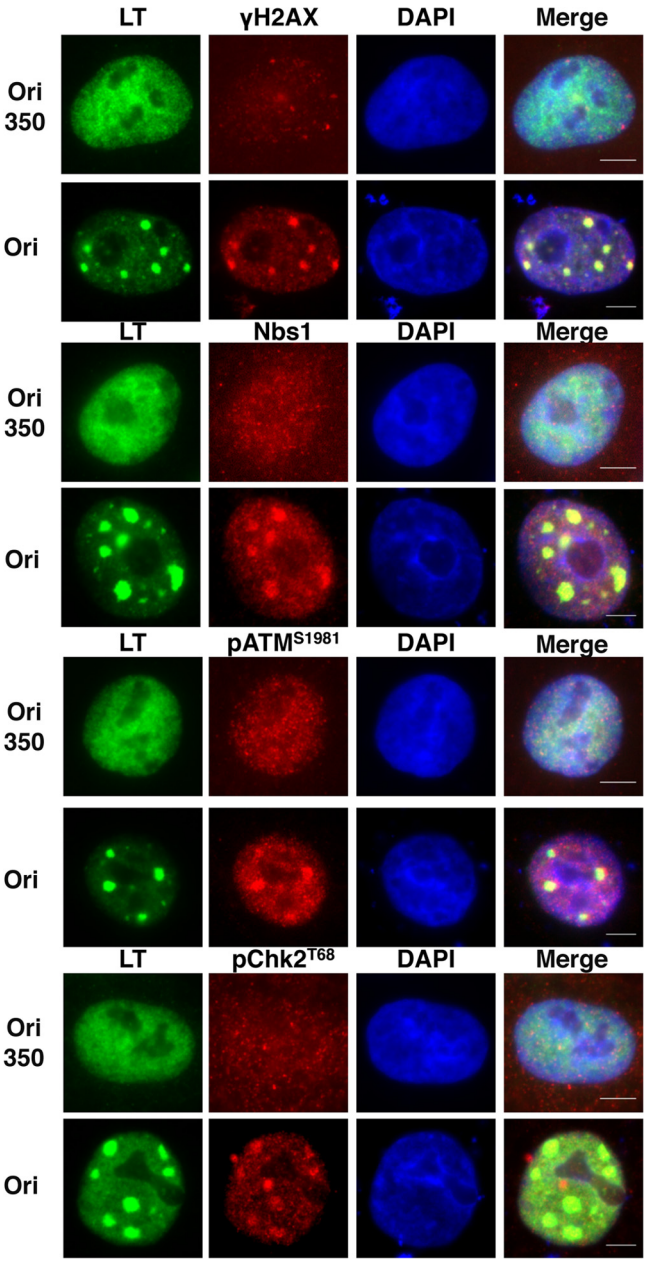

FIG 3 ATM-mediated DDR machinery colocalizes with MCPyV LT in nuclear foci. C33A cells were cotransfected with a plasmid carrying MCPyV LT and with a plasmid carrying either the wild-type MCPyV origin (Ori) or the replication-defective origin (Ori350). At 48 hpt, cells were stained for MCPyV LT (green) and DDR proteins (red), as indicated. The cells were counterstained with DAPI. Representative pictures from at least three experiments are shown. Bars, 5 μm.

mediated pathway. Similar to what we observed with components of the ATM-mediated DDR, components of the ATR-mediated pathway also colocalized with LT in nuclear foci (Fig. 4). ATR and pChk1^{S317} were observed to colocalize with LT nuclear foci in 95.8% ± 0.4% and 80.2% ± 7.8% of cells displaying LT foci, respectively. In response to DNA damage, RPA32, the 32-kDa subunit of RPA, is also hyperphosphorylated by ATM, ATR, and DNA-dependent protein kinase (DNA-PK) to contribute to repair DNA synthesis (37). We also detected the signal of RPA32^{S33} phosphorylation at the LT nuclear foci in 100% of cells displaying LT foci (Fig. 4). Because the factors from both ATM- and ATR-mediated DDR pathways did not accumulate in distinct foci in cells cotransfected with an MCPyV LT-encoding construct and a control vector carrying the SV40 Ori (data not shown), these data suggest that the DDR factors are associated with MCPyV LT at nuclear foci in an MCPyV Ori-dependent manner. C33A cells cotransfected with wild-type LT and Ori350 also displayed a diffuse pattern for both LT and various DDR factors (Fig. 3 and 4), further supporting the suggestion that the formation of DDR protein/LT foci depends on the LT-mediated replication of MCPyV DNA.

We also performed Western blot analysis to examine the activation status of the ATM- and ATR-mediated DDR pathways in C33A cells transfected with MCPyV LT together with or without MCPyV Ori (Fig. 5). Consistent with our previous observations in U2OS cells (14), activation of the ATR pathway (as indicated by the induction of Chk1^{S345} phosphorylation) was observed in C33A cells expressing MCPyV LT, either in the presence or in the absence of MCPyV Ori (Fig. 5A). MCPyV LT expression also appeared to moderately induce ATM^{S1981} phosphorylation in C33A

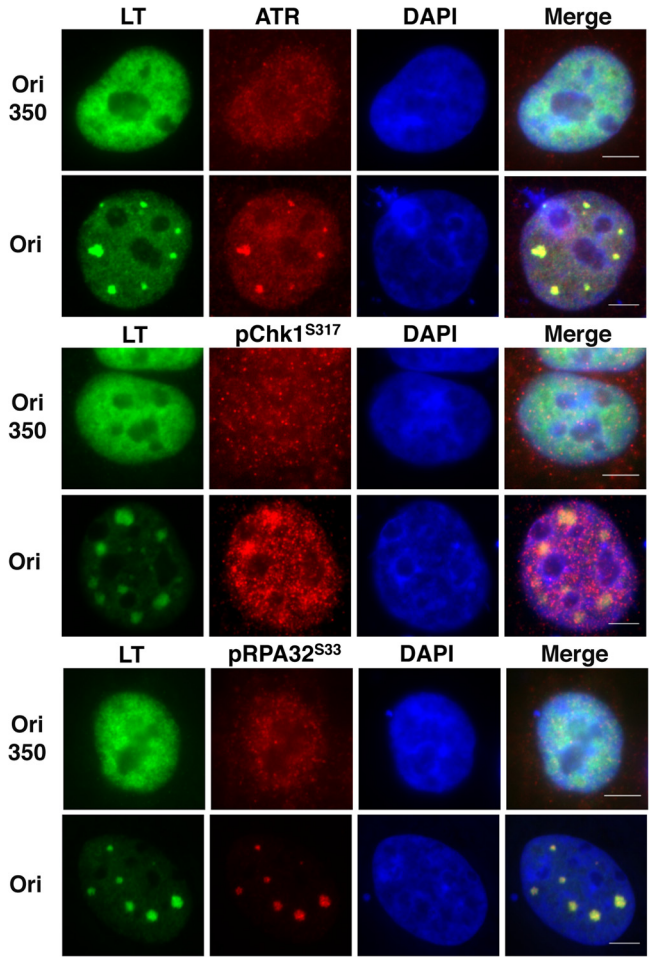

FIG 4 ATR-mediated DDR machinery colocalizes with MCPyV LT in nuclear foci. C33A cells were transfected as described in the legend to Fig. 3. At 48 hpt, cells were stained for MCPyV LT (green) and DDR proteins (red), as indicated. The cells were counterstained with DAPI. Representative pictures from at least three experiments are shown. Bars, 5 μm.

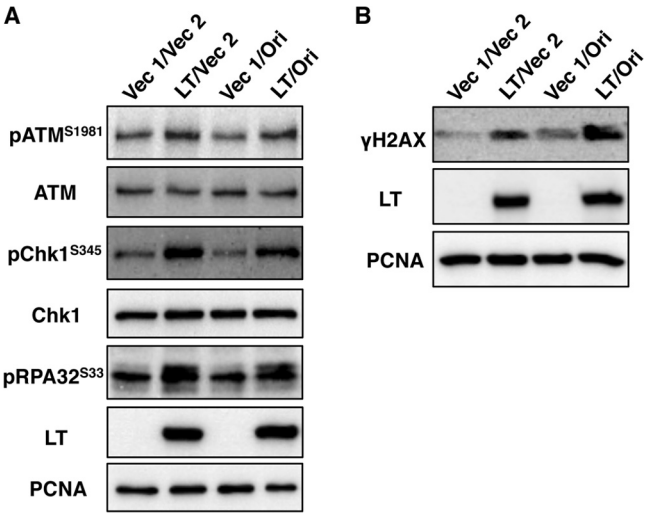

FIG 5 MCPyV LT activates a host DDR in C33A cells. C33A cells were cotransfected with a plasmid carrying either MCPyV LT or the control vector (Vec 1) and with a plasmid carrying either the MCPyV origin (Ori) or an empty vector (Vec 2). At 48 hpt, nuclear proteins were harvested for Western blotting with the indicated antibodies for ATM/ATR activation markers (A) or for γH2AX (B). PCNA was used as a loading control for nuclear proteins.

cells, indicating the activation of ATM kinase in these cells (Fig. 5A). In addition, C33A cells transfected with MCPyV LT either with or without MCPyV Ori also showed increased RPA32^{S33} phosphorylation and an increased γH2AX signal (Fig. 5). These results demonstrate that the ATM- and ATR-mediated DDR pathways are activated in C33A cells expressing MCPyV LT.

Host DDR machinery and MCPyV LT colocalize at actively replicating viral origins in the nucleus. Previously, we have detected DNA damage in U2OS cells upon ectopic expression of full-length MCPyV LT or C-terminus-only LT truncation mutants (14). It is possible that the DDR machinery is recruited to the sites of DNA breaks on the host genome. To ensure that the DDR factor/LT nuclear foci that we observed in C33A cells are, in fact, at the MCPyV Ori and not sites of repair on the host genome, we performed immuno-FISH to detect the localization of MCPyV Ori with respect to the host DDR machinery. C33A cells were cotransfected with a plasmid carrying MCPyV LT and a different plasmid carrying the MCPyV Ori. We then detected the localization of LT and DDR proteins by immunofluorescent staining and used a specific probe to detect the MCPyV Ori-containing plasmid by FISH (Fig. 6A). The FISH signal was detected only with the MCPyV Ori-specific probe and not with a negative-control probe recognizing the HPV16 genome, which is absent from the HPV-negative C33A cells (Fig. 6A). Consistent with our previous observation (12), MCPyV LT and MCPyV Ori colocalized in nuclear foci (Fig. 6A, top), which we believe to be MCPyV replication factories containing actively amplifying viral DNA and not single copies of the origin (see below). In addition, the various components of the host DDR machinery, such as ATR, pChk2^{T68}, and γH2AX, also colocalized with the MCPyV Ori foci (Fig. 6A and data not shown). These data demonstrate the recruitment of DDR proteins to the MCPyV Ori complex, confirming that the nuclear foci that we observed are not the result of LT-induced DNA damage on the host chromosomes. Our results also suggest that the DDR proteins may be involved in MCPyV DNA replication.

To rule out the possibility of nuclear aggregation and to ensure that the nuclear foci that we observed in the presence of MCPyV Ori are, in fact, the sites of viral replication, we tested for BrdU incorporation at these foci. Previously, we have used this technique to demonstrate the incorporation of BrdU specifically in MCPyV LT/Ori foci (12). Consistently, in cells cotransfected with MCPyV LT and the Ori, the BrdU signal was observed in distinct nuclear foci, whereas in the control samples, the BrdU signal was minimal (Fig. 6B). Interestingly, both ATR and pChk2^{T68} were found to colocalize with the BrdU signal (Fig. 6B), suggesting that the DDR factor/LT foci that we observed are indeed sites of active, robust viral DNA replication. Nevertheless, it is also possible that cells without these replication foci are maintaining low levels of MCPyV replication that are undetectable by immuno-FISH and BrdU incorporation analysis.

Formation of DDR protein/MCPyV LT nuclear foci is viral replication dependent. To confirm that the nuclear foci at which MCPyV LT and DDR factors colocalize are, in fact, dependent on viral DNA replication, we employed the U2OS 2-6-3 system, in which a LacO array has been integrated into the cellular genome of U2OS cells (38). U2OS 2-6-3 cells were transfected with a plasmid

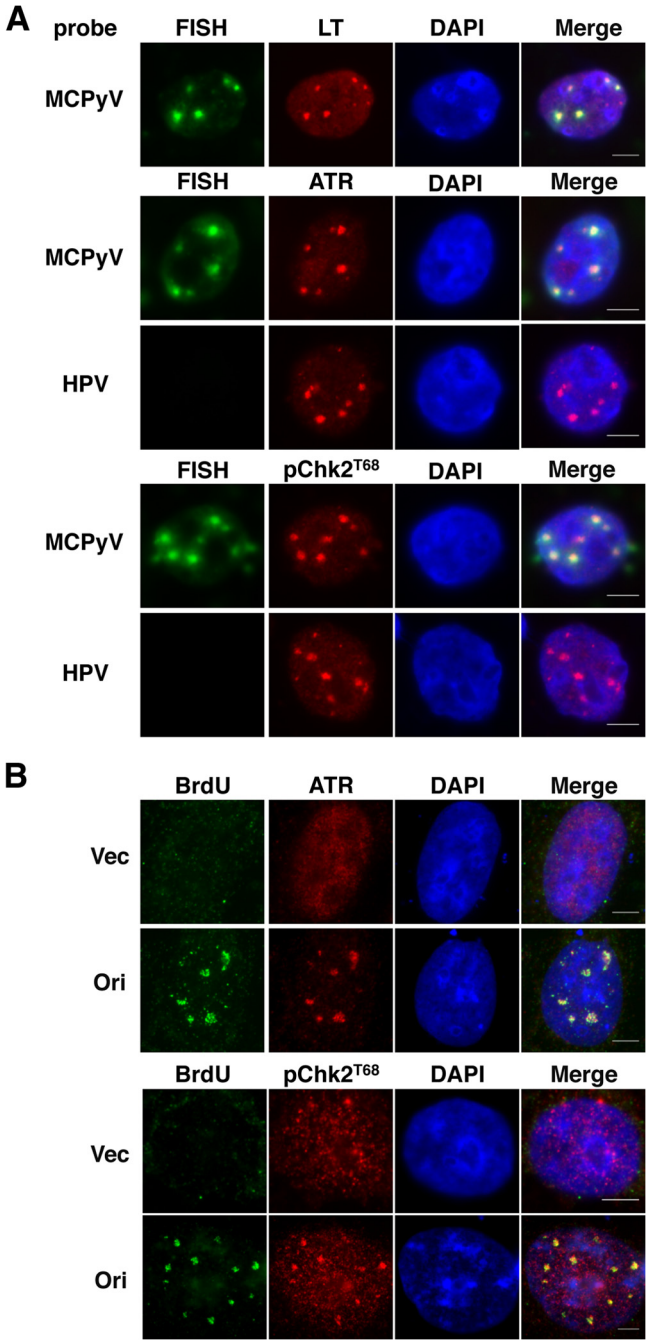

FIG 6 DDR proteins localize at the replicating MCPyV origin in the nucleus. (A) C33A cells were cotransfected with a plasmid carrying MCPyV LT and a plasmid carrying the MCPyV origin (Ori). At 60 hpt, cells were stained for MCPyV LT and DDR proteins (red), as indicated. A specific probe recognizing the MCPyV Ori plasmid and a nonspecific probe recognizing the HPV genome were used for FISH (green). The cells were counterstained with DAPI. Representative pictures from at least three experiments are shown. Bars, 5 μm. (B) C33A cells were cotransfected with a plasmid carrying MCPyV LT and with a plasmid carrying either the MCPyV origin (Ori) or an empty vector (Vec). At 44 hpt, cells were pulsed with BrdU for 2 h. The cells were incubated in regular medium for another 2 h before they were fixed and stained for BrdU (green) and DDR proteins (red), as indicated. The cells were counterstained with DAPI. Representative pictures from at least three experiments are shown. Bars, 5 μm.

carrying LacR-fused MCPyV LT. Consequently, the LT expressed in these cells was tethered to the LacO array due to the tight binding of LacR to LacO, which was seen as a single LT-positive focus in each cell (Fig. 7A, LT). We immunostained LacR-LT-transfected U2OS 2-6-3 cells for various DDR proteins to see if LT itself physically interacts and brings these DDR factors to the LacO array. However, we observed no colocalization of LT and DDR proteins in these cells, suggesting that LT itself, even though it is concentrated in a single focus within the nucleus, cannot tether host DDR proteins to the LacO site. Considering the high affinity of LacR to LacO and the fact that the genome of U2OS 2-6-3 cells transfected with LacR alone cannot replicate normally (data not shown), we believe that the binding of LacR to LacO is so tight that this particular locus cannot be unwound/replicated. The absence of DDR factor/LT colocalization in this system suggests that the focus formation seen in Fig. 1 to 4 and 6 is replication dependent.

In addition, we constructed a number of MCPyV LT mutants on the basis of homology to SV40 LT helicase mutations that abolish SV40 replication. When we cotransfected C33A cells with wild-type LT and the wild-type viral Ori, we could robustly detect LT-mediated replication of the Ori plasmid by Southern blotting (Fig. 7B, Newly synthesized DNA). However, when MCPyV LT helicase mutants were transfected in place of the wild-type LT, replication of the Ori plasmid was completely abolished, confirming that these LT mutants were, in fact, replication defective. The mutants were expressed at levels comparable to those of wild-type LT (Fig. 7B, LT), indicating that the failure to replicate the wild-type Ori plasmid was not due to the gross instability of the mutants. DNA digested with only BamHI showed comparable hybridization of the Southern blot probe to the Ori-containing plasmid (Fig. 7B, Loading control), demonstrating comparable transfection efficiency and loading. The Southern blotting results correlated with a dramatic reduction in LT focus formation (quantified in Fig. 7C). This provides additional evidence for the notion that the formation of LT foci depends on productive replication of viral DNA.

Moreover, wild-type LT cotransfected with Ori350 showed a much more attenuated ability to replicate the plasmid carrying the mutated Ori (Fig. 7B), and this also correlated with the decrease in the number of LT foci quantified (Fig. 7C). These data from LT mutants and the Ori350 mutant support the suggestion that the DDR protein/LT foci observed in our system are, in fact, viral replication dependent.

Treatment with DDR inhibitors reduces MCPyV DNA replication. Next, we tested the functional importance of the host DDR to MCPyV by studying the effects of DDR inhibitors on autonomous MCPyV DNA replication. Wortmannin was used in this study because it is a potent, covalent inhibitor of PI3Ks (39), which include ATM, ATR, and DNA-PK. However, it has been reported that wortmannin inhibits DSB repair and not SSB repair (40) and that at a higher concentration (20 μM) it efficiently inhibits ATM activation *in vivo* (41). Therefore, we also tested the effects of NU6027, which specifically inhibits ATR activity but not ATM or DNA-PK activity (42), on MCPyV DNA replication. We first confirmed by Western blot analysis that wortmannin can reduce etoposide-induced ATM^{S1981} phosphorylation and that NU6027 can inhibit UVC-induced Chk1^{S345} phosphorylation (data not shown). We then tested these drugs in C33A cells transfected with the pT+Ori plasmid carrying the sequences of MCPyV LT, sT, and the viral Ori, which has been shown to sup-

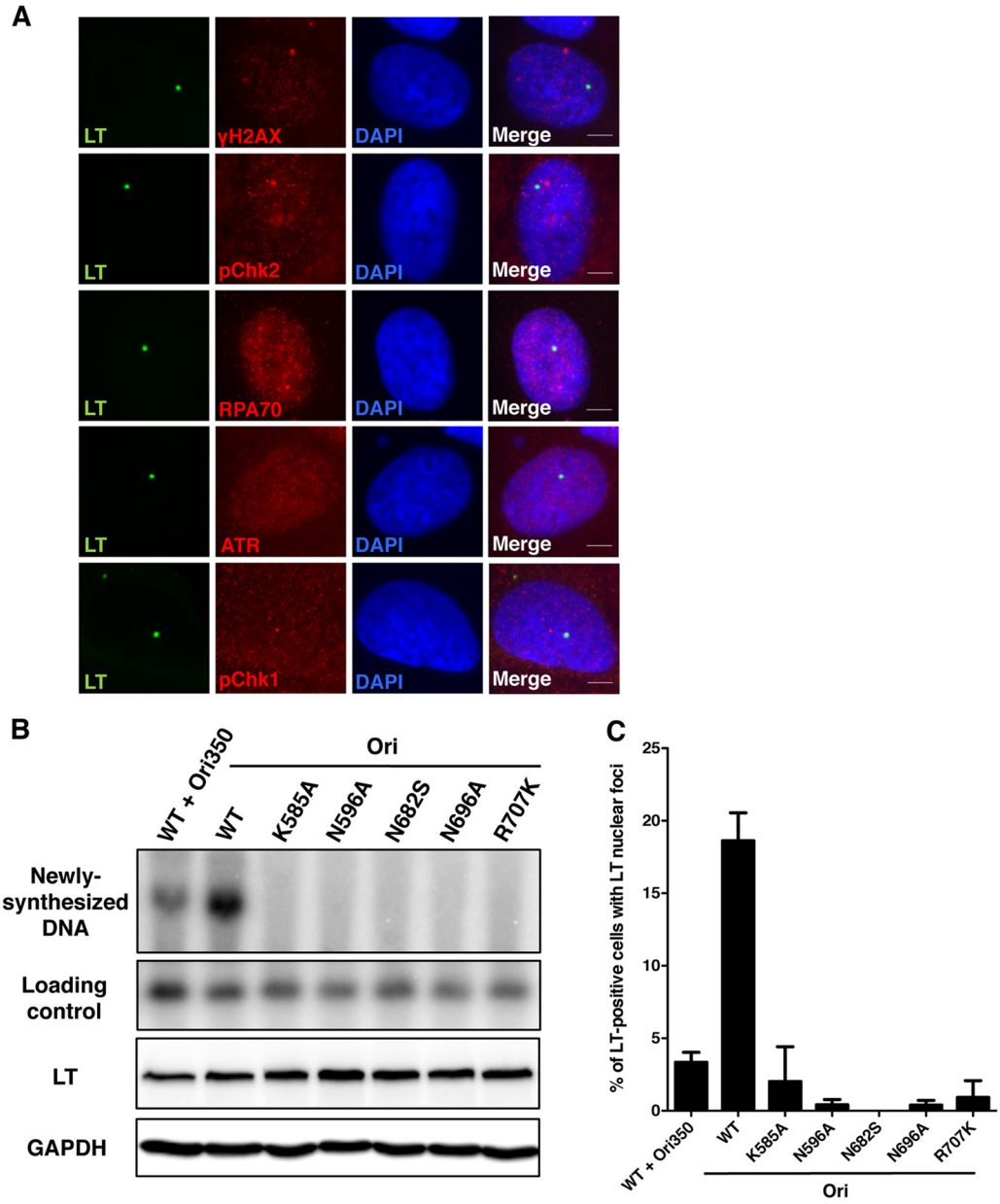

FIG 7 Formation of DDR protein/LT nuclear foci is dependent on MCPyV DNA replication. (A) U2OS 2-6-3 cells were transfected with a plasmid carrying LacR-fused MCPyV LT. At 36 hpt, cells were stained for MCPyV LT (green) and DDR proteins (red), as indicated. The cells were counterstained with DAPI. Representative pictures from at least three experiments are shown. Bars, 5 μm. (B) C33A cells were cotransfected with a plasmid carrying wild-type (WT) or mutant MCPyV LT (LT) and with a plasmid carrying either the wild-type MCPyV origin (Ori) or the replication-defective origin (Ori350). At 48 hpt, total cellular DNA was extracted for Southern blotting. Fifteen micrograms of DNA was digested with BamHI and DpnI to detect replicated origin plasmid, while 2 μg of DNA was digested with only BamHI to show equal loading. Total protein extractions were used in Western blotting to detect MCPyV LT and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). (C) C33A cells were transfected as described for panel B. At 48 hpt, cells were stained for MCPyV LT as described in the legend to Fig. 3. About 150 LT-positive cells were quantified for the presence of LT nuclear foci. Data represent means and standard deviations calculated from three independent experiments.

port autonomous replication in our previous study (12). In untreated and DMSO-treated C33A cells, robust pT+Ori replication was detected (Fig. 8). In contrast, in the DDR inhibitor-treated samples, we noticed a decrease in pT+Ori replication (Fig. 8A and B). This reduction was particularly drastic in wortmannin-treated C33A cells (Fig. 8A and B), possibly due to wortmannin's multitargeting of phosphatidylinositol 3-kinases (see Discussion). Both wortmannin and NU6027 treatments also efficiently inhibited the replication of the religated MCV genome (data not shown). Consistent with the Southern blotting results, we observed a decrease in replication focus formation in drug-treated cells by immunofluorescent staining (data not shown). We believe that the decrease in autonomous replication is a consequence of DDR inhibition, as drug treatment had a minimal effect on MCPyV LT expression (Fig. 8A).

We also performed flow cytometry analysis to test how wort-

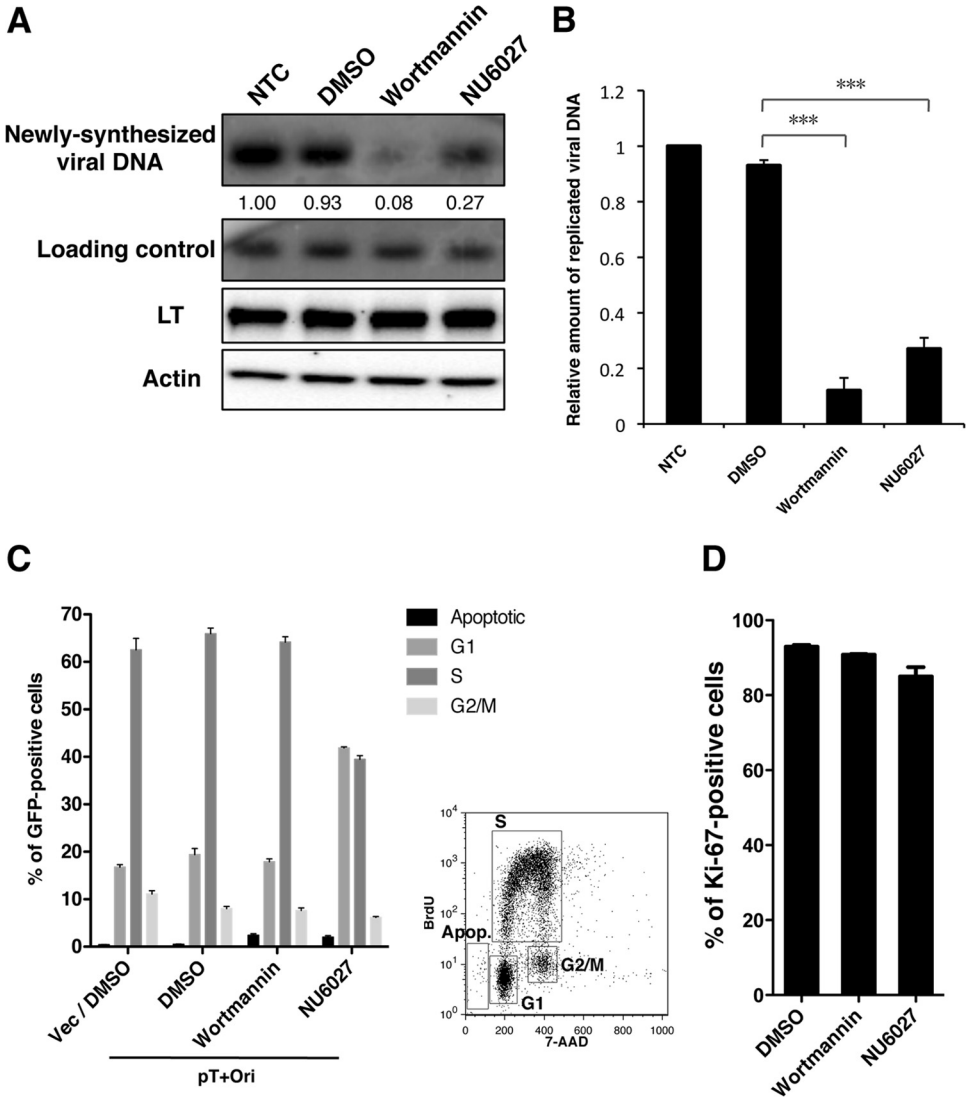

FIG 8 Treatment with wortmannin or NU6027 reduces viral DNA replication. C33A cells were transfected with pT+Ori carrying MCPyV LT, sT, and the viral origin. At 20 hpt, cells were treated with DMSO, 20 μM wortmannin, or 20 μM NU6027. DMSO and the DDR inhibitors were refreshed every 16 h. (A) At 52 hpt, total cellular DNA was extracted for Southern blotting. Thirty micrograms of DNA was digested with XhoI and DpnI to detect replicated viral DNA, while 2 μg of DNA was digested with only XhoI to show equal loading. Quantification was normalized to the amount for the nontreated control (NTC). Total protein extractions were used in Western blotting to detect MCPyV LT and actin. (B) Quantification of replicated viral DNA bands. The value from the nontreated control was arbitrarily set to 1. Data represent means and standard deviations calculated from three independent experiments. ***, $P < 0.001$. (C) C33A cells were transfected with pT+Ori or an empty vector (Vec). Cells were drug treated as described for panel A. At 50 hpt, cells were pulsed with BrdU for 2 h and then fixed and stained with BrdU antibody and 7-aminoactinomycin D (7-AAD). About 12,000 GFP-positive cells were analyzed by flow cytometry. A representative gating strategy for each phase of the cell cycle is shown. Data represent means and standard deviations calculated from three independent experiments. (D) C33A cells were transfected and drug treated, as described for panel A. At 52 hpt, cells were stained for Ki-67. About 200 cells were quantified for Ki-67 positivity. Data represent means and standard deviations calculated from three independent experiments.

mannin and NU6027 affect the host cell cycle, which could in turn affect pT+Ori replication. Although wortmannin treatment had a minimal effect on the cell cycle profile, NU6027 treatment led to an increase in the G_1 population compared to that for the pT+Ori-transfected, DMSO-treated control (Fig. 8C). Even though we detected an ~70% decrease in pT+Ori replication upon NU6027 treatment (Fig. 8B), part of this reduction could be a consequence of G_1 arrest in C33A cells. We further tested if drug treatment affects cellular proliferation by immunostaining drug-treated C33A cells for Ki-67, which is an established marker for cell proliferation detected in all active phases of the cell cycle (G_1, S, and G_2/M) but not in resting cells (G_0). As shown in Fig. 8D, neither DDR inhibitor had much of an effect on Ki-67 positivity. This demonstrates that the drug-treated cells were, in fact, actively proliferating.

siRNA knockdown of ATR and/or ATM inhibits MCPyV DNA replication. To rule out the possibility that wortmannin and NU6027 have off-target and/or cell cycle effects on C33A cells that could affect MCPyV DNA replication, we performed siRNA knockdown of ATR and/or ATM to see if we could recapitulate observations with the DDR inhibitors. siRNA knockdown of ATR or ATM inhibited the activation of Chk1^{S345} and Chk2^{T68} phos-

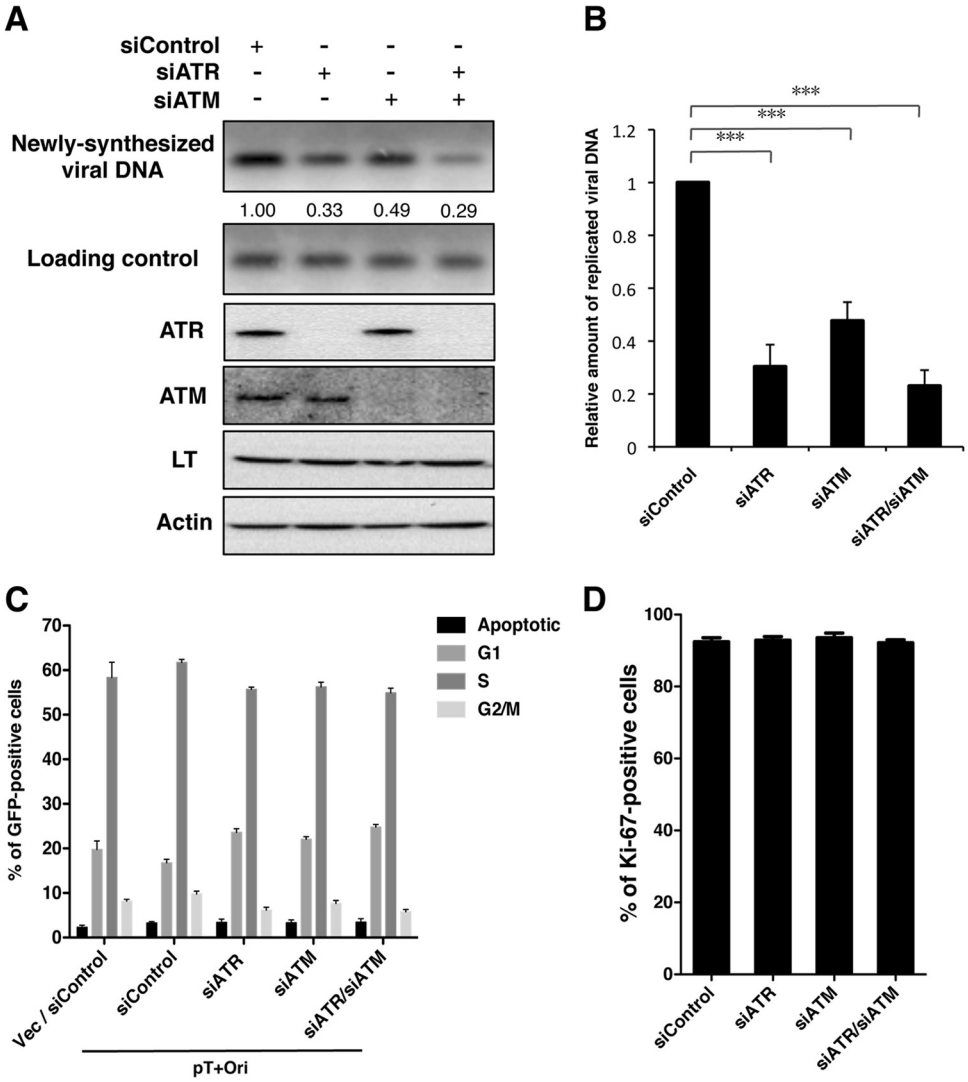

FIG 9 siRNA knockdown of ATR and ATM reduces viral DNA replication. C33A cells were cotransfected with pT+Ori and control siRNA (siControl), siATR, and/or siATM. (A) At 48 hpt, total cellular DNA was extracted for Southern blotting. Thirty micrograms of DNA was digested with XhoI and DpnI to detect replicated viral DNA, while 2 μg of DNA was digested with only XhoI to show equal loading. Quantification was normalized to the amount for control siRNA. Total protein extractions were used in Western blotting for ATR, ATM, MCPyV LT, and actin. (B) Quantification of replicated viral DNA bands. The value from control siRNA was arbitrarily set to 1. Data represent means and standard deviations calculated from three independent experiments. ***, $P < 0.001$. (C) C33A cells were cotransfected with the indicated siRNA and either pT+Ori or an empty vector (Vec). At 46 hpt, cells were pulsed with BrdU for 2 h and then fixed and stained with BrdU antibody and 7-aminoactinomycin D. About 12,000 GFP-positive cells were analyzed by flow cytometry. Data represent means and standard deviations calculated from three independent experiments. (D) C33A cells were transfected as described for panel A. At 48 hpt, cells were stained for Ki-67. About 200 cells were quantified for Ki-67 positivity. Data represent means and standard deviations calculated from three independent experiments.

phorylation, respectively, in cells treated with UVC (data not shown). We cotransfected pT+Ori with control siRNA, siRNA against ATR (siATR), and/or siRNA against ATM (siATM) into C33A cells and performed Southern blotting to detect newly synthesized pT+Ori. At 48 hpt, both ATR and ATM were effectively knocked down (Fig. 9A). Similar to what we have observed with the DDR inhibitors, ATR and ATM knockdown reduced autonomous pT+Ori replication in C33A cells (Fig. 9A and B). This decrease was even more apparent when both ATR and ATM were knocked down (Fig. 9A and B). We were also able to observe a decrease in replication focus formation in ATR/ATM double-knockdown cells by immunofluorescent staining (data not shown). The fact that ATR/ATM double-knockdown cells showed a slightly greater reduction in pT+Ori replication than ATR or ATM single-knockdown cells could be explained by the functional redundancy of these two kinases. The decrease in pT+Ori replication is attributed to the knockdown of ATR and ATM, as siRNA treatment had a minimal effect on MCPyV LT expression (Fig. 9A), host cell cycle (Fig. 9C), and cellular proliferation (Fig. 9D) in C33A cells. These data suggest that both ATR- and ATM-mediated DDR is important for optimal MCPyV DNA replication.

DISCUSSION

Since its discovery, MCPyV has provided a new model for studying the oncogenic potential of polyomaviruses in humans. Although there is evidence suggesting that the interactions between

LT and host cell cycle-regulatory proteins contribute to tumor development (14, 15), the exact oncogenic mechanism of MCPyV has not yet been established. Currently, we have limited understanding of the MCPyV life cycle, including viral entry, replication, and propagation processes. Recent reports on MCPyV receptor usage (10, 36, 43) and tropism (11) have begun to shed light on MCPyV basic biology. Our study of the host factors required for MCPyV replication has also started to tease out the mechanistic details of viral genome amplification (12). Using our established system for the study of MCPyV DNA replication (12), we investigated additional host machinery that is important for this process.

In this study, we showed that, in cells infected with MCPyV, components of the ATM and ATR kinase pathways accumulate in MCPyV LT-positive nuclear foci. Colocalization of these DDR factors and MCPyV LT in nuclear foci was also observed in cells either transduced with pseudotyped virions composed of the HPV capsid and MCPyV genome or transfected with the MCPyV genome. This suggests that gene expression from the MCPyV genome and not incoming virion-associated proteins is responsible for inducing the formation of DDR factor/LT nuclear foci. Using our previously established immuno-FISH method to visualize MCPyV LT/Ori replication complexes in cells (12), we showed that the DDR proteins and MCPyV LT colocalize at complexes that contain actively replicating MCPyV DNA. We believe that focus formation represents the presence of robust viral DNA replication, where the newly synthesized DNA has accumulated to a level detectable by immuno-FISH/BrdU staining. It is possible that cells without these replication factories are maintaining low levels of MCPyV replication. While the immunofluorescent staining revealed the formation of MCPyV DNA replication factories in the presence of robust replication, Southern blot analysis was used to demonstrate the overall level of MCPyV replication maintained in cells with and without replication factories. Abrogation of the host DDR, using either chemical inhibitors (wortmannin and NU6027) or ATR/ATM siRNA knockdown, led to a decrease in MCPyV DNA replication, supporting the conclusion that these components of the host DDR are essential for robust viral genome amplification.

To date, it has been technically challenging to study the MCPyV life cycle in cultured cell lines due to the lack of a known natural host cell and the inefficient replication of the native viral genome in culture (reviewed in references 44 and 45). In our initial experiments, we were able to detect accumulation of DDR factors in the MCPyV LT-positive nuclear foci in a limited number of cells infected with native MCPyV virions. To further investigate the involvement of host DDR in MCPyV replication, our current study relied on an MCPyV DNA replication system that we previously established (12), in which MCPyV LT and Ori plasmids were transiently transfected into C33A cells. Although this system suggests that the DDR machinery is important for MCPyV replication, it is essential to investigate how DDR activation contributes to viral replication in more natural MCPyV replication systems once they are established. Currently, there is some uncertainty about the nature of the precursor cells that give rise to MCC tumors (46). Understanding the behavior of MCPyV in MCC precursor cell types will be important for further elucidation of the oncogenic effects of the virus.

In our study, we used the PI3K inhibitor wortmannin and ATR inhibitor NU6027 to show that the DDR response contributes to MCPyV replication. Since wortmannin not only inhibits ATM and ATR but also inhibits other members of the PIKK family, such as DNA-dependent protein kinase (DNA-PK), we also tested a number of other DDR inhibitors, including NU7441 (a DNA-PK inhibitor), KU55933 (an ATM inhibitor), and AZD7762 (a Chk1 inhibitor). However, none of these drug treatments showed as dramatic of an effect as wortmannin and NU6027 (data not shown). This suggests that ATR may be more important for MCPyV replication than other DDR mediators. In line with the findings of the experiments performed with drug inhibitors (Fig. 8 and data not shown), assays with siRNA showed a more dramatic reduction in LT-mediated replication when ATR was knocked down than when ATM was knocked down, while ATR and ATM double knockdown decreased replication to the level similar to that obtained with ATR single knockdown (Fig. 9A and B).

Many groups have previously reported the activation of ATR- and/or ATM-dependent DDR pathways upon viral infection or viral protein expression. HPV, adenovirus, herpes simplex virus, Epstein-Barr virus, and retroviruses all induce a DDR (47, 48). So far, research on polyomaviruses has also shown similar phenomena. However, despite the large amount of information on the activation of host DDR by many viruses, little is known about the mechanism by which these viruses trigger such a response in the host. Previously, SV40 LT alone has been shown to induce both ATR- and ATM-mediated DDR, and this DDR activation is dependent on LT's interaction with the mitotic spindle checkpoint kinase Bub1 (30). In addition, for JCPyV, there is evidence suggesting that its LT-mediated G_2 cell cycle arrest is dependent on LT's ability to associate with cellular DNA (25). This observation leads to the speculation that perhaps the viral origin-binding domain and the nonspecific DNA-binding domains on LT could tether this viral helicase to host chromosomes, allowing it to unwind host DNA, which in turn would trigger DDR complex recruitment to the cellular DNA. This idea is in line with a recent report on BKPyV that showed severe chromosomal damage upon BKPyV infection in the absence of ATR/ATM (26). Moreover, studies have shown that SV40 and BKPyV infection leads to the accumulation of a cell population with >4N DNA content (26). For MCPyV, it is possible that the DNA intermediates generated during viral DNA replication are recognized by the host cells as damaged DNA, which could trigger the DDR activation that we observed upon viral infection/replication.

One of the most interesting questions remains: how exactly do viruses benefit from the activation of DDR? As mentioned above, ATR- and ATM-mediated DDR can help repair chromosomal damage caused by polyomavirus infection (26). This repair mechanism not only allows the host to sustain virus-induced DNA damage but also permits the virus to propagate without killing the host. For SV40 and JCPyV, there is evidence suggesting that the activation of ATM- and ATR-mediated checkpoint signaling leads to cell cycle arrest in S and G_2 phases, which are conducive to viral replication (24, 25). We have also observed that MCPyV LT-induced ATR activation can lead to a modest G_2 arrest (14), suggesting that the response may contribute to viral DNA replication by inducing a cellular environment that is beneficial for viral DNA replication. This model is consistent with a recent report from Cheng and colleagues showing that full-length LT can trigger growth inhibition in cultured cells (49). On the other hand, our immunofluorescent staining showed little ATR/ATM accumulation outside viral replication factories, so the virus-induced host DNA damage and repair are likely minimal. Furthermore, because

the components of the ATM and ATR pathways localize to the sites of MCPyV replication, which contain replication factors and actively replicating viral DNA (12; this study), the DDR proteins are likely playing a more direct role in MCPyV DNA replication. For example, the host DDR factors may promote viral DNA replication by repairing the viral replication-induced damage on its own DNA (27).

How these DDR proteins contribute to MCPyV DNA replication will be the focus of our future study. Activation and recruitment of DDR factors have also been observed during HPV replication (50–53). For HPV, it is thought that the host machinery used for homologous recombination may be required for the circularization of the viral genome for virion packaging (51). Interestingly, our recent studies have demonstrated that the cellular protein Brd4 is recruited to both HPV and MCPyV replication complexes to contribute to viral DNA replication (12, 33). Brd4 has been shown to interact with the DNA damage response protein ATAD5 (54, 55), suggesting that it may play a role in DNA damage repair-associated viral DNA replication. Future studies will investigate whether Brd4 is involved in recruiting DDR factors and DNA damage-specific polymerases to the HPV and MCPyV origin to support DDR-mediated viral replication.

Most of the studies on polyomavirus replication have been performed using Southern blotting, quantitative PCR, or *in vitro* assays. The study presented in this report combined immunofluorescent staining, immuno-FISH, and BrdU incorporation to visualize the MCPyV replication complexes in cells. Building upon this platform, our study demonstrates that the host's DDR proteins are important for robust MCPyV DNA replication. This system will be useful for further investigation of the mechanistic role of DDR factors in MCPyV replication. For example, excessive UV exposure is a major risk factor for MCC. Future study will investigate whether overstimulation of host DDR by sunlight exposure may cause abnormal viral DNA replication, leading to viral DNA integration and oncogenic progression. Collectively, not only do these studies on the relationship between the host DDR and MCPyV provide insight into the host machinery required for MCPyV genome amplification, but also they may shed light on virus-associated oncogenesis. Therefore, research on the components of the host DDR may have important clinical implications for MCPyV-associated MCC.

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